

## Surface charge engineering of PQQ glucose dehydrogenase for downstream processing

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### Abstract

The ion-exchange chromatography behavior of recombinant glucose dehydrogenase harboring pyrroloquinoline quinone (PQQGDH) was modified to greatly simplify its purification. The surface charge of PQQGDH was engineered by either fusing a three-arginine tail to the C-terminus of PQQGDH (PQQGDH+Arg3) or by substituting three residues exposed on the surface of the enzyme to Arg by site-directed mutagenesis (3RPQQGDH). During cation exchange chromatography, both surface charge-engineered enzymes eluted at much higher salt concentrations than the wild-type enzyme. After the chromatography purification step, both PQQGDH+Arg3 and 3RPQQGDH appeared as single bands on SDS-PAGE, while extra bands appeared with the wild-type protein sample. Although all tested kinetic parameters of both engineered enzymes are similar to those of wild type, both modifications resulted in enzymes with increased thermal stability. Our achievements have resulted in the greater production of an improved quality PQQGDH by a simplified process.

### Introduction

The glucose dehydrogenase possessing pyrroloquinoline quinone as the prosthetic group (PQQGDH) is recognized as an ideal enzyme for mediator-type glucose sensors. Because the electrochemical regeneration of its cofactor is not affected by the presence of oxygen in the samples (Kost *et al.* 1998, Tang *et al.* 2001), it is considered as the alternative to the conventionally used glucose oxidase (GOD). However, several properties of PQQGDH remain inferior to GOD, such as its stability and substrate specificity. The authors have been engaged in the engineering of PQQGDH in order to improve its enzymatic properties (Igarashi *et al.* 1999, Sode *et al.* 2000, 2002, Igarashi & Sode 2003).

The availability of a cost-effective recombinant production system for an industrial enzyme is essential to have a superior product on the market. The recombinant expression of PQQGDH of *Acinetobacter*

*calcoaceticus* using *E. coli* as the host strain was first reported by Cleton-Jansen *et al.* (1989). Since *E. coli* cannot synthesise PQQ, recombinant PQQGDH in *E. coli* was produced as an apo enzyme. We previously reported the production of recombinant PQQGDH using an *E. coli* strain harboring a heterologous *pqq* operon from *Klebsiella pneumoniae* to produce holo enzymes in the presence of bivalent metal in the medium (Sode *et al.* 1996). We have also reported the secretional production of PQQGDH using *Pichia pastoris* as the host microorganism (Kojima *et al.* 2000).

Besides, concentration and purification of recombinant products in the initial steps would reduce the recovery costs. Although target proteins are often fused to affinity tags to simplify downstream processing, this may not always be acceptable. Such fusions may result in new proteolytic cleavage sites, decreases in enzymatic activity, or even in formation of inclusion bodies. Furthermore, the introduction of

certain affinity tags requires the use of expensive affinity resins. The addition of charged amino acid residues to the C-terminus is an alternative engineering method to simplify the purification of recombinant proteins with cost-effective ion exchange chromatography. The C-terminal fusions of both cationic peptides such as poly-arginine (Graslund *et al.* 2000, 2002, Sassenfeld & Brewe 1984, Zhang & Glatz 1999) and anionic peptides such as poly-aspartate (Le Borgne 1995, Stubenrauch 2000, Zhang *et al.* 2001) have been reported. The fusion of such small peptides did not affect enzymatic properties, however, they drastically altered the ion-exchange chromatography behavior, thus reducing further purification steps.

In this study, we report the engineering of ion-exchange chromatography behavior of PQQGDH by either fusing poly-arginine, herein after Arg-tail, or by substituting appropriate residues to Arg by site-directed mutagenesis, to greatly simplify the purification procedure of recombinant PQQGDH.

## Materials and methods

### Strains and plasmids

*E. coli* PP2418, in which the PQQGDH structural gene was disrupted by insertion mutagenesis (Cleton-Jansen *et al.* 1990), was used as the host strain for the expression of each PQQGDH. *E. coli* BMH71-18mutS and *E. coli* MV1184 were used for constructing mutations by site-directed mutagenesis. All PQQGDH structural genes were inserted into the multi-cloning site of the expression vector pTrc99A (Pharmacia, Sweden).

### Genetic manipulation

#### Construction of PQQGDH+Arg3

PQQGDH with a 3-Arg tail at the C-terminus (PQQGDH+Arg3) was created by amplifying the PQQGDH structural gene (*gdh-B*) by PCR with the following program: {96 °C 10 min, [96 °C 4 min; 60 °C 1 min; 72 °C 1.5 min (30 times)], 72 °C 10 min}. The pGB vector (Sode *et al.* 2000), into which the PQQGDH structural gene was inserted, was used as the template and the pair of oligonucleotides below were used as the primers, as summarized in Figure 1.

Forward: 5'-GGCCATGGATAAACATTATTGGCTAAATTGCTTTAT-3'

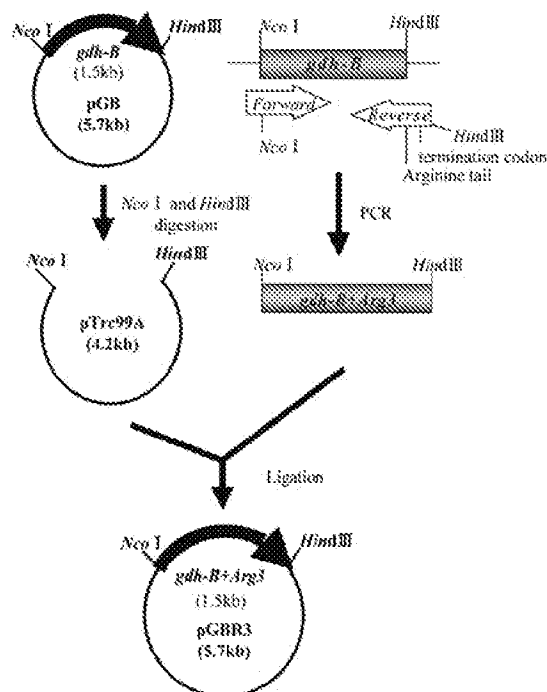


Fig. 1. Construction of PQQGDH+Arg3, a PQQGDH with an arginine tail at the C-terminus.

Reverse: 5'-CCAAGCTTTTACCTTCGACGCTTAGCCTTATAGGTGAACTTAATGAG-3'

To facilitate cloning, *Nco*I and *Hind*III restriction enzyme sites (*italics*) were incorporated into the forward and reverse primers, respectively. The reverse primer was also designed to introduce three consecutive arginine residues (bold and underlined) followed by a termination codon (bold) to the 3' end of the PQQGDH open reading frame. The resulting PCR product (*gdh-B*+Arg3) was digested with *Nco*I and *Hind*III and inserted into the multi-cloning site of pTrc99A vector digested with the same enzymes. The sequence of the inserted gene fragment was confirmed by automated DNA sequencing (ABI Model 310; Applied Biosystems, USA), and the constructed vector was designated as pGBR3.

#### Construction of 3RPQQGDH

For the internal substitutions to Arg, we selected residues that are located on the surface of PQQGDH with side chains exposed to solvent but that have no effect on the enzymatic properties. Gln209, Asn240,

and Thr389 were chosen for substitution to Arg (see results).

A 1.2-kbp *KpnI-HindIII* fragment from pGB, containing most of the PQQGDH gene, was inserted into *KpnI*- and *HindIII*-digested pKF18k (TaKaRa Bio Inc., Japan). The resulting construct was used to carry out site-directed mutagenesis using Mutan-Express Km (TaKaRa Bio Inc., Japan) according to the manufacturer's instructions with the oligonucleotide primers shown below.

Gln209Arg: 5'-GCCAACTCAACGTGAACCTGAATG-3'

Asn240Arg: 5'-CCAAGTTTTCGCGGGGTGGTTAG-3'

Thr389Arg: 5'-GTAAAAAAGCAATTCGTGGTTGGGAAAATAC-3'

The nucleotide sequence of this mutant was also confirmed by automated DNA sequencing. After digestion with *KpnI* and *HindIII*, the mutated PQQGDH gene fragment was substituted into the corresponding region of pGB. The resulting expression vector containing the mutated PQQGDH (3RPQQGDH) was named pGB3R.

#### *Production and purification of wild-type PQQGDH, PQQGDH+Arg3, and 3RPQQGDH*

*E. coli* PP2418 was transformed with pGB, pGBR3, or pGB3R and grown in 7 l of L broth supplemented with ampicillin (25  $\mu\text{g ml}^{-1}$ ) and chloramphenicol (30  $\mu\text{g ml}^{-1}$ ) at 37 °C for 2.5 h, until an OD<sub>660</sub> of 2 was reached. The cells were induced with 0.3 mM (final concentration) of IPTG and the incubation was continued for 4 h at 30 °C. The approximately 7 g cells harvested by centrifugation were resuspended in 10 ml of 10 mM MOPS/NaOH buffer, pH 7. Polyethyleneimine P-70 (PEI; Wako Pure Chemical Industries, Ltd., Japan) was added to give 0.25% (w/v) to the resuspended cells harboring pGBR3 or pGB3R. To the cells harboring pGBR3, ZnCl<sub>2</sub> was also added to a final concentration of 0.5 mM. Resuspended cells were then disrupted by four passages through a French Pressure cell at 110 MPa followed by ultra-centrifugation (160 500  $\times g$ , 1.5 h, 4 °C). The supernatants were dialyzed overnight at 4 °C against 10 mM MOPS/NaOH buffer, pH 7, containing 1 mM CaCl<sub>2</sub>, followed by centrifugation to remove any precipitate that might have formed. The resulting crude extract of each enzyme was applied to a CM-5pw cation exchange

column (Tosoh Corporation, Japan) equilibrated with 10 mM MOPS/NaOH buffer, pH 7, containing 1 mM CaCl<sub>2</sub>. After washing the column with the same buffer until a stable UV baseline was obtained, the enzyme was eluted with a linear gradient of 0 to 0.32 M NaCl in the same buffer. The fractions showing GDH activity were dialyzed against 10 mM MOPS/NaOH buffer at 4 °C. The purified mutant enzymes, which appeared as single silver-stained bands on SDS-PAGE, were utilized for the measurement of kinetic parameters and thermal stability. Wild-type PQQGDH required additional purification steps, as previously reported (Sode *et al.* 2000), to achieve a comparable purity level.

#### *Measurements of enzymatic activity and SDS-PAGE analysis*

Each purified enzyme sample was incubated in a total volume of 200  $\mu\text{l}$  of 10 mM MOPS/NaOH buffer, pH 7, for 10 min at 25 °C in the presence of 1  $\mu\text{M}$  PQQ and 1 mM CaCl<sub>2</sub> to form a holo-enzyme. Phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCIP) were then added to final concentrations of 0.6 mM and 0.06 mM, respectively. Upon addition of the substrate, the rate of decrease of absorbance at 600 nm was measured spectrophotometrically. One unit of enzymatic activity is defined as the reduction of 1  $\mu\text{mol}$  DCIP min<sup>-1</sup>, using the molar extinction coefficient for DCIP at pH 7 of 16.3 mM<sup>-1</sup>. Protein concentrations of samples were measured by DC Protein Assay Kit II (Bio-Rad Laboratories Inc., USA) according to the manufacturer's instructions.

SDS-PAGE was performed on 8–25% gradient gels with Phast System (Amersham Biosciences, UK) according to the manufacturer's instructions.

#### *Analysis of thermal stability*

The thermal stabilities of the various PQQGDHs were evaluated as the residual activity of the enzyme as a function of incubation time at 55 °C. Holo enzyme samples described above were incubated at 55 °C for various durations, followed by 2 min on ice, 10 min at 25 °C, and measurement of GDH activity in the presence of 100 mM glucose.

Substrate specificities were evaluated by the measurements of the kinetic parameters for glucose, allose, 3-*O*-methyl-glucose, galactose, lactose and maltose. Each substrate was added at various concentrations after holo enzyme formation and  $V_{\text{max}}$  and  $K_m$  values were calculated for each enzyme.

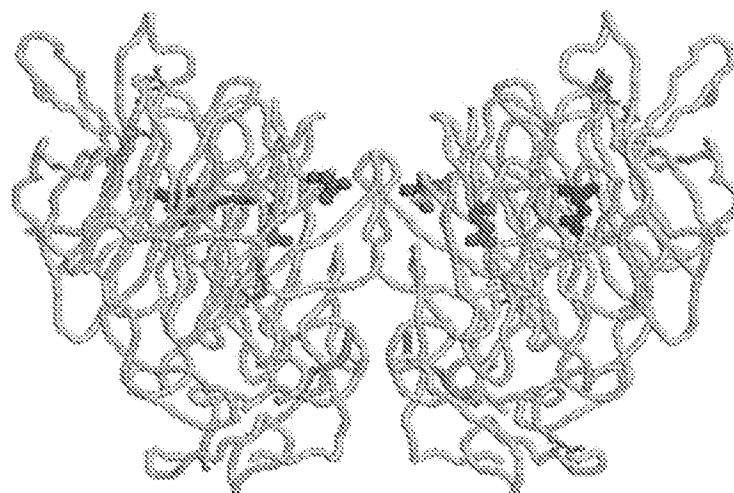


Fig. 2. 3-D model of PQQGDH, showing the residues substituted to create 3RPQQGDH. Gln209, Asn240, Thr389, *N*-terminus, and *C*-terminus are shown as ball and stick model in dimeric PQQGDH structure (PDB code: 1QBI with slight modification).

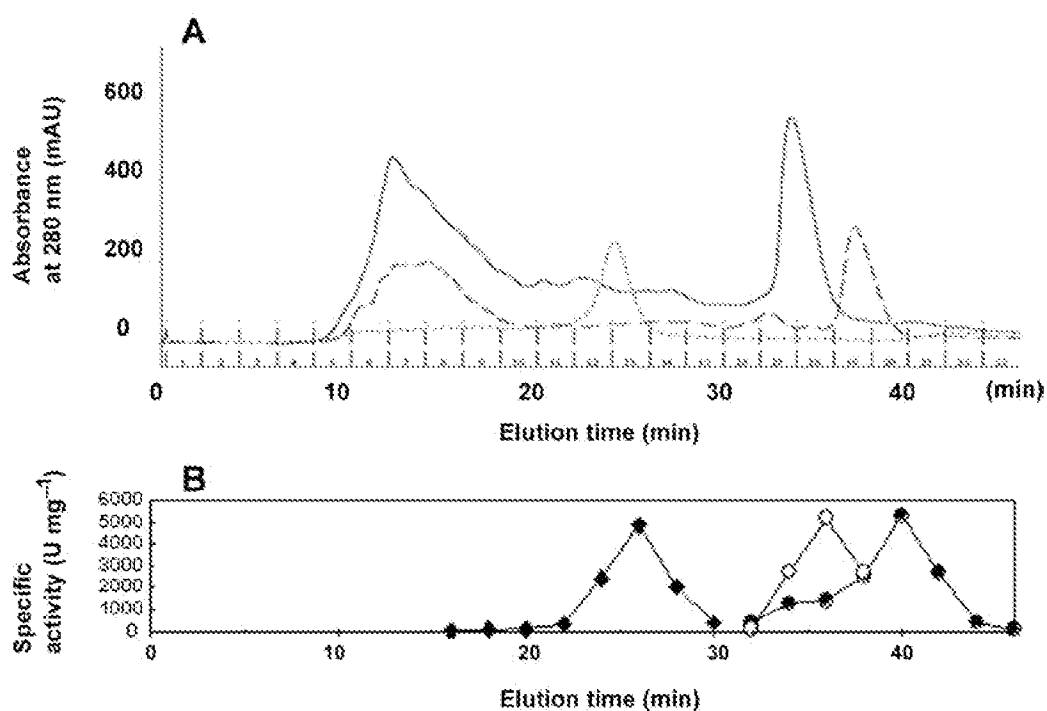


Fig. 3. Results of cation exchange chromatography of PQQGDHs on CM-5pw column. (A) Chromatograms of wild-type PQQGDH (dotted line), PQQGDH+Arg3 (dashed line), and 3RPQQGDH (solid line). (B) Relationship between elution time and GDH activity of wild-type PQQGDH (◆), PQQGDH+Arg3 (●), and 3RPQQGDH (○).

## Results

### Designing of 3RPQQGDH

Figure 2 shows the 3-D model of the engineered PQQGDH with increased surface positive charge. We selected amino acid residues to be substituted that meet the following 3 criteria: 1) residues with side chains exposed to the solvent, 2) polar uncharged residues, and 3) residues that are not involved in functional regions such as catalytic site or dimer interface. Also taking into account the codons of the amino acid candidates, we selected Gln209, Asn240, and Thr389 to be substituted to Arg. Since PQQGDH is a dimeric enzyme, substituting these 3 residues resulted in the introduction of 6 Arg per enzyme dimer.

### Purification of engineered enzymes

Figure 3 shows the elution profiles (A) and the corresponding PQQGDH activities (B) for wild-type PQQGDH, PQQGDH+Arg3, and 3RPQQGDH. The wild-type PQQGDH eluted in approximately 80 mM NaCl, while PQQGDH+Arg3 and 3RPQQGDH eluted at much higher salt concentrations, at 210 mM and 190 mM, respectively. In each case, the enzyme elution was confirmed by the presence of a major activity peak matching with the corresponding major protein peak. A minor active peak of PQQGDH+Arg3 also eluted at a slightly lower salt concentration, probably due to partial degradation of the Arg tail.

After the cation exchange chromatography purification step, both PQQGDH+Arg3 and 3RPQQGDH appeared as single bands on SDS-PAGE, while the wild-type enzyme sample contained considerable quantities of extra proteins (Figure 4). Shifting the enzyme elution profile by increasing the surface positive charge therefore seems to have improved the purification step, eliminating the requirement for additional purification steps.

### Characterization of engineered enzymes

The kinetic parameters of wild-type PQQGDH, PQQGDH+Arg3, and 3RPQQGDH were determined with a number of substrates, as summarized in Table 1. The  $K_m$  and  $V_{max}$  values of wild-type PQQGDH with glucose (27 mM and 7040 U mg<sup>-1</sup>, respectively) were almost identical to those of PQQGDH+Arg3 ( $K_m$  = 27 mM,  $V_{max}$  = 7040 U mg<sup>-1</sup>) and 3RPQQGDH ( $K_m$  = 25 mM,  $V_{max}$  = 7120 U mg<sup>-1</sup>).  $K_m$  and  $V_{max}$  values with the other substrates were also very

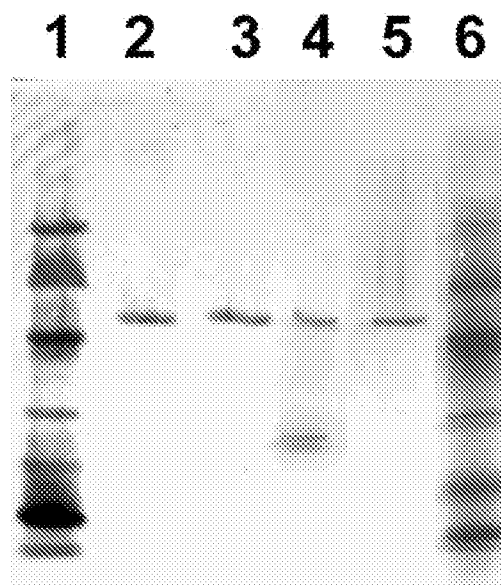


Fig. 4. SDS-PAGE analysis of PQQGDHs. Low-molecular-weight marker (lanes 1 and 6) consists of phosphorylase b (97-kDa), albumin (66-kDa), ovalbumin (45-kDa), carbonic anhydrase (30-kDa), trypsin inhibitor (20.1-kDa) and  $\alpha$ -lactalbumin (14.4-kDa) (Amersham Biosciences, UK). PQQGDH+Arg3 (lane 2), 3RPQQGDH (lane 3), wild-type PQQGDH (lane 4), and completely purified wild-type PQQGDH (lane 5).

similar in all three enzymes investigated, indicating that the catalytic and substrate binding sites were not significantly affected by the mutations.

Figure 5 shows the thermal inactivation time course of the three PQQGDHs at 55 °C. The wild-type enzyme inactivated at this temperature with a half-life of approx. 20 min. In contrast, both PQQGDH+Arg3 and 3RPQQGDH showed almost identical rates of decay, showing much greater thermal stability than the wild type, with half-lives of approx. 70 min. Therefore, the introduction of positive charges on the surface of PQQGDH resulted in unexpected, yet welcomed, increases in the thermal stability.

## Discussion

We have now simplified the purification of PQQGDH by engineering the enzyme's surface charge to shift its elution profile during cation exchange chromatography. As demonstrated on the chromatogram (Figure 4), the introduction of positively charged amino acid residues shifted the elution to much higher salt concentrations. The separation of the mutant PQQG-

Table 1. Kinetic parameters of PQQGDHs for various substrates.

	Wild-type			PQQGDH+Arg3			3RPQQGDH		
	$K_m$ (mM)	$V_{max}$ (U mg <sup>-1</sup> )	$V_{max}/K_m$ (U mg <sup>-1</sup> mM)	$K_m$ (mM)	$V_{max}$ (U mg <sup>-1</sup> )	$V_{max}/K_m$ (U mg <sup>-1</sup> mM)	$K_m$ (mM)	$V_{max}$ (U mg <sup>-1</sup> )	$V_{max}/K_m$ (U mg <sup>-1</sup> mM)
Glucose	27	7040	261 (100%)	27	7040	261 (100%)	25	7120	285 (100%)
Allose	63	3810	61 (23%)	75	4680	62 (24%)	76	5370	71 (25%)
3- <i>O</i> -m-glucose <sup>a</sup>	41	5110	125 (48%)	53	6440	122 (47%)	56	6670	119 (42%)
Galactose	2	323	162 (62%)	4	256	64 (25%)	5	304	61 (21%)
Lactose	25	3710	148 (57%)	26	2870	110 (42%)	36	3560	99 (35%)
Maltose	10	2020	202 (77%)	11	1860	169 (65%)	13	2370	182 (64%)

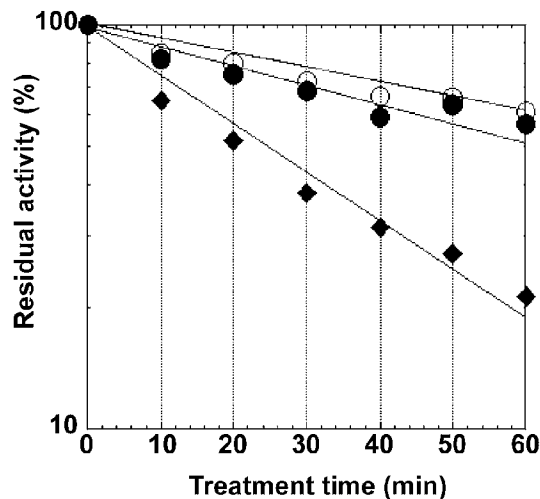
<sup>a</sup>3-*O*-methylglucose.

Fig. 5. Thermal stability of PQQGDHs at 55 °C. Wild-type PQQGDH (◆), PQQGDH+Arg3 (●), and 3RPQQGDH (○) were incubated at 55 °C. Aliquots were taken every 10 min to measure residual activity.

DHs from other *E. coli* proteins is thereby greatly improved, resulting in a pure protein after a single purification step. Moreover, the genetic modifications did not alter the catalytic activity or substrate specificity; however, they improved the enzyme's thermal stability.

There are several reported attempts at engineering proteins for improving the suitability of the downstream processing. Among the several approaches, changing the surface charge of a protein is a very promising technique since the engineered proteins are purified by ion exchange chromatography, which does not require specially designed and expensive affinity resin. In this paper, we introduced a cationic tail composed of 3 Arg residues, which, as expected,

shifted the elution of the protein to a much higher salt concentration. Unfortunately, addition of the positively charged tail resulted in the elution of minor peaks of PQQGDH, probably due to partial degradation of the tail. Several proteases have been reported to prefer degrading positively charged C-terminal regions. These proteases exist in periplasmic space of *E. coli* and are inhibited by ZnCl<sub>2</sub>. To minimize such proteolytic degradation of the Arg tail, the purification of PQQGDH+Arg3 was therefore carried out in the presence of 0.5 mM ZnCl<sub>2</sub>. The absence of ZnCl<sub>2</sub> resulted in the appearance of much significant fraction containing PQQGDH activity (results were not shown). Considering these complications, internal substitutions to cationic residues at appropriate positions, as was achieved in 3RPQQGDH, appears preferable since no degradation was detected.

Both PQQGDH+Arg3 and 3RPQQGDH show similar kinetic parameters as wild-type PQQGDH. This was achieved by selecting positions for mutagenesis that have no important functional roles. The C-terminal region does not interact with the dimer interface or active site. Similarly, the three residues substituted in 3RPQQGDH are on the surface distant from the active site and dimer interface.

An unexpected increase in thermal stability was observed for both PQQGDH+Arg3 and 3RPQQGDH. The introduction of such positively charged polar residues on the surface of the enzyme may result in a decrease of the formation of hydrophobic interactions, thus decreasing the irreversible formation of protein aggregation. Alternatively, the improved thermal stability may be due to the differences in purification. Minor impurities, absent in the purified mutant samples, might cause inactivation of the wild-type enzyme.

PQQGDH is an industrially important enzyme that required the development of a simple and efficient process to yield high quality enzyme to be used in diagnostic tools. Our current achievement has advanced the development of a processing method for the industrial production of this enzyme.

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